

### Claims

1. A method of forming gradient loaded liposomes having a lower inside/higher outside pH gradient, the method comprising:
  - 5 (a) contacting a solution of liposomes with a pharmaceutical agent in an aqueous solution of up to about 60 mM of an acid, at a temperature wherein the protonated form of the pharmaceutical agent is charged and is not capable of permeating the membrane of the liposomes, and wherein the unprotonated form of the pharmaceutical agent is uncharged and is capable of permeating the membrane  
10 of the liposomes;
  - (b) cooling the solution to a temperature at which the unprotonated form of the pharmaceutical agent is not capable of permeating the membrane of the liposomes; and
  - (c) contacting the solution with a weak base, in an amount effective to raise  
15 the pH of the internal liposome to provide gradient loaded liposomes having a lower inside/higher outside pH gradient.
2. The method of claim 1 wherein the liposomes comprise phosphatidylcholine.  
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3. The method of claim 1 wherein the liposomes comprise phosphatidylcholine selected from the group of distearoylphosphatidylcholine, hydrogenated soy phosphatidylcholine, hydrogenated egg phosphatidylcholine, dipalmitoylphosphatidylcholine, dimyristoylphosphatidylcholine, and dielaidoyl  
25 phosphatidyl chline.
4. The method of claim 1 wherein the liposomes further comprise cholesterol.
5. The method of claim 1 wherein the liposomes further comprise  
30 phosphatidylglycerol.

6. The method of claim 1 wherein the liposomes further comprise non-phosphatidyl lipids.
7. The method of claim 6 wherein the non-phosphatidyl lipids comprise sphingomyelin.
8. The method of claim 1 wherein the liposomes further comprise phosphatidylglycerol selected from the group of dimyristoylphosphatidylglycerol, dilaurylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, and distearoylphosphatidylglycerol.
9. The method of claim 1 wherein the liposomes comprises phosphatidylcholine, and further comprises cholesterol.
10. The method of claim 1 wherein the liposomes comprises phosphatidylcholine, and further comprises cholesterol, wherein the molar ratio of the phosphatidylcholine to the cholesterol is about 1:0.01 to about 1:1.
11. The method of claim 1 wherein the liposomes comprises phosphatidylcholine, and further comprises cholesterol, wherein the molar ratio of the phosphatidylcholine to the cholesterol is about 1.5:1.0 to about 3.0:1.0.
12. The method of claim 1 wherein the liposomes are unilamellar and less than about 100nm.
13. The method of claim 1 wherein the weight ratio of the liposomes to the pharmaceutical agent is up to about 200:1.
14. The method of claim 1 wherein the weight ratio of the liposomes to the pharmaceutical agent is about 1:1 to about 100:1.

15. The method of claim 1 wherein the weight ratio of the liposomes to the pharmaceutical agent is about 1:1 to about 50:1.
16. The method of claim 1 wherein the acid has an acid dissociation constant of less than about  $1 \times 10^{-2}$ .
17. The method of claim 1 wherein the acid has an acid dissociation constant of less than about  $1 \times 10^{-4}$ .
18. The method of claim 1 wherein the acid has an acid dissociation constant of less than about  $1 \times 10^{-5}$ .
19. The method of claim 1 wherein the acid has a permeability coefficient larger than about  $1 \times 10^{-4}$  cm/sec for the liposomes.
20. The method of claim 1 wherein the acid is selected from the group of formic acid, acetic acid, propanoic acid, butanoic acid, pentanoic acid, citric acid, oxalic acid, succinic acid, lactic acid, malic acid, tartaric acid, fumaric acid, benzoic acid, aconitic acid, veratric acid, phosphoric acid, sulfuric acid, and combinations thereof.
21. The method of claim 1 wherein the acid is citric acid.
22. The method in claim 1 wherein up to about 50 mM of an acid is employed.
23. The method of claim 1 wherein the pharmaceutical agent exists in a charged state when dissolved in an aqueous medium.
24. The method of claim 1 wherein the pharmaceutical agent is an organic compound that includes at least one acyclic or cyclic amino group, capable of being protonated.

25. The method of claim 1 wherein the pharmaceutical agent is an organic compound that includes at least one primary amine group, at least one secondary amine group, at least one tertiary amine group, at least one quaternary amine group, or any combination thereof.

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26. The method of claim 1 wherein the pharmaceutical agent is an antineoplastic agent.

27. The method of claim 1 wherein the pharmaceutical agent is a combination of  
10 two or more antineoplastic agents.

28. The method of claim 1 wherein the pharmaceutical agent is an ionizable basic antineoplastic agent.

15 29. The method of claim 1 wherein the pharmaceutical agent is an anthracycline chemotherapeutic agent, an anthracenedione, an amphiphilic drug, or a vinca alkaloid.

30. The method of claim 29 wherein the anthracycline chemotherapeutic agent is  
20 selected from the group of doxorubicin, epirubicin, and daunorubicin.

31. The method of claim 29 wherein the anthracenedione is mitoxantrone.

32. The method of claim 29 wherein the amphiphilic drug is a lipophilic amine.  
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33. The method of claim 29 wherein the vinca alkaloid is selected from the group of vincristine and vinblastine.

34. The method of claim 1 wherein the pharmaceutical agent is an antineoplastic  
30 antibiotic.

35. The method of claim 1 wherein the pharmaceutical agent is not camptothecin, or an analogue thereof.
36. The method of claim 1 wherein the pharmaceutical agent is an alkylating agent.
37. The method of claim 36 wherein the alkylating agent is selected from the group of cyclophosphamide and mechlorethamine hydrochloride.
38. The method of claim 1 wherein the pharmaceutical agent is a purine or pyrimidine derivative.
39. The method of claim 38 wherein the purine or pyrimidine derivative is 5-fluorouracil.
40. The method of claim 1 wherein the temperature in step (a) is about 40°C to about 70°C.
41. The method of claim 1 wherein the temperature in step (a) is about 50°C to about 60°C.
42. The method of claim 1 wherein the solution is cooled in step (b) to a temperature of about 0°C to about 30°C.
43. The method of claim 1 wherein the solution in step (a) is prepared by the process comprising:
- (i) contacting the liposomes and the aqueous solution of the acid;
  - (ii) homogenizing the solution; and
  - (iii) optionally removing any external acid.

44. The method of claim 43 wherein the external acid is removed in step (iii) by filtering the external acid.
45. The method of claim 1 wherein the weak base is a membrane permeable amine.
46. The method of claim 1 wherein the weak base is an ammonium salt or an alkyl amine.
47. The method of claim 1 wherein the weak base is an ammonium salt having a mono- or multi-valent counterion.
48. The method of claim 1 wherein the weak base is selected from the group of ammonium sulfate, ammonium hydroxide, ammonium acetate, ammonium chloride, ammonium phosphate, ammonium citrate, ammonium succinate, ammonium lactobionate, ammonium carbonate, ammonium tartarate, ammonium oxalate, and combinations thereof.
49. The method of claim 1 wherein the weak base is alkyl-amine selected from the group of methyl amine, ethyl amine, diethyl amine, ethylene diamine, and propyl amine.
50. The method of claim 1 further comprising, during or after step (c), removing any unloaded pharmaceutical agent.
51. The method of claim 50 wherein the removing of the unloaded drug employs removing the unloaded drug via cross filtration or dialysis.
52. The method of claim 1 further comprising, after step (c), dehydrating the liposomes.

53. The method of claim 52 wherein the dehydrating is carried out at a pressure of below about 1 atm.

54. The method of claim 52 wherein the dehydrating is carried out with prior  
5 freezing of the liposomes.

55. The method of claim 52 wherein the dehydrating is carried out in the presence of one or more protective monosaccharide sugars, one or more protective disaccharide sugars, or a combination thereof.

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56. The method of claim 55 wherein the protective sugar is selected from the group of trehalose, sucrose, maltose, and lactose.

57. The method of claim 52 further comprising rehydrating the liposomes after  
15 the dehydrating.

58. The method of claim 1 wherein the liposomes are unilamellar vesicles.

59. The method of claim 1 wherein the liposomes are multilamellar vesicles.  
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60. The method of claim 1 wherein more than about 90 wt.% of the pharmaceutical agent is trapped in the liposomes.

61. The method of claim 1 further comprising, after step (c), contacting the  
25 liposomes with a pharmaceutically acceptable carrier.

62. The method of claim 1 wherein the acid is present in about 20 mM to about 60 mM.

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63. A method for preparing a pharmaceutical composition comprising:
- (a) contacting a solution of liposomes with a pharmaceutical agent in an aqueous solution of up to about 60 mM of an acid, at a temperature wherein the protonated form of the pharmaceutical agent is charged and is not capable of permeating the membrane of the liposomes, and wherein the unprotonated form of the pharmaceutical agent is uncharged and is capable of permeating the membrane of the liposomes;
  - (b) cooling the solution to a temperature at which the unprotonated form of the pharmaceutical agent is not capable of permeating the membrane of the liposomes;
  - (c) contacting the solution with a weak base, in an amount effective to raise the pH of the internal liposome to provide gradient loaded liposomes having a lower inside/higher outside pH gradient; and
  - (d) combining the liposomes with a pharmaceutically acceptable carrier to provide the pharmaceutical composition.
64. A method comprising administering the pharmaceutical composition of claim 63 to a mammal.
65. A method for treating a mammal inflicted with cancer, the method comprising administering the pharmaceutical composition of claim 63 to the mammal, wherein the pharmaceutical agent is an antineoplastic agent.
66. The method of claim 65 wherein the cancer is a tumor, ovarian cancer, small cell lung cancer (SCLC), non small cell lung cancer (NSCLC), leukemia, sarcoma, colorectal cancer, head cancer, neck cancer, or breast cancer.
67. The method of claim 65 wherein the administration of the antineoplastic agent, *via* the liposomal formulation, has a toxicity profile that is lower than the toxicity profile associated with the administration of the antineoplastic agent in the free form.



68. The method of claim 67 wherein the toxicity is selected from the group of gastrointestinal toxicity and cumulative dose-dependent irreversible cardiomyopathy.

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69. The method of claim 65 wherein the administration of the antineoplastic agent has unpleasant side-effects that are lower in incidence, severity, or a combination thereof, than unpleasant side-effects associated with the administration of the antineoplastic agent in the free form.

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70. The method of claim 69 wherein the unpleasant side-effects are selected from the group of myelosuppression, alopecia, mucositis, nausea, vomiting, and anorexia.

15 71. A gradient loaded liposome having a lower inside/higher outside pH gradient prepared by the process comprising:

(a) contacting a solution of liposomes with a pharmaceutical agent in an aqueous solution of up to about 60 mM of an acid, at a temperature wherein the protonated form of the pharmaceutical agent is charged and is not capable of permeating the membrane of the liposomes, and wherein the unprotonated form of the pharmaceutical agent is uncharged and is capable of permeating the membrane of the liposomes;

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(b) cooling the solution to a temperature at which the unprotonated form of the pharmaceutical agent is not capable of permeating the membrane of the liposomes; and

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(c) contacting the solution with a weak base, in an amount effective to raise the pH of the internal liposome to provide gradient loaded liposomes having a lower inside/higher outside pH gradient.

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